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Patulin-producing molds in corn silage and high moisture corn and effects of patulin on fermentation by ruminal microbes in continuous culture

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Abstract

The objectives were to investigate the presence of patulin-producing *Penicillium* sp. in corn silage and high moisture corn as well as adverse effects of patulin on microbial fermentation in continuous culture fermenters. Eighty-three samples of corn silage or high moisture corn were cultured to determine the presence of molds. *Penicillium* sp. were isolated from 0.82 of samples. Of these *Penicillium* sp. isolates, 0.03 produced patulin on yeast extract sucrose and potato dextrose agar. The patulin-producing isolates belonged to the *P. viridicatum* group. The other molds identified were: *Mucor* sp. (0.45), *Aspergillus* sp. (0.41), and *Fusarium* sp. (0.25). Eight single-flow continuous culture fermenters were used to study effects of patulin on fermentation by ruminal microbes. Two 1-l fermenters were supplemented with 0, 10, 20 or 40 mg of patulin every 12 h for three consecutive days. Increasing patulin reduced neutral detergent and acid detergent fiber digestibility at a decreasing rate

Abbreviations: APDA, acid potato dextrose agar; cfu/g, colony forming units per gram; CYA, Czapeck yeast autolysate; PDA, potato dextrose agar; YES, yeast extract sucrose; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; TNC, total non-structural carbohydrates; VFA, volatile fatty acid; NH_3-N, ammonia nitrogen; NAN, non-ammonia nitrogen; EMPS, efficiency of microbial protein synthesis; BCVFA, branched-chain VFA; ENU, efficiency of N utilization

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(linear, $P < 0.01$; quadratic, $P < 0.05$). True digestion of organic matter and total non-structural carbohydrates decreased linearly ($P < 0.05$) as patulin concentration increased. Crude protein digestion and bacterial N flows decreased linearly ($P < 0.05$). Conversely, there was a linear increase ($P < 0.05$) in ammonia nitrogen with increased patulin. Total, ammonia and non-ammonia N flows were not affected by patulin. Efficiency of microbial protein synthesis was not affected by patulin but there was a linear decreased ($P < 0.05$) in the efficiency of N utilization. Increasing patulin levels caused a linear decrease ($P < 0.001$) of total volatile fatty acid concentration and a quadratic decrease of acetate and propionate molar proportions ($P < 0.05$). Ten and 20 mg/l of patulin produced a decrease in acetate proportion and an increase in propionate proportion. Lactate concentration (mmol/l) increase from 0.0 to 216.5 mmol/l (linear, $P < 0.05$) with increasing patulin concentration. *Penicillium* sp. molds are common contaminants of corn silage and high moisture corn and they produce patulin that can adversely affect fermentation by ruminal microbes. Alterations in microbial digestion of dry matter, and production of microbial end products, impact the production and/or health of ruminants.

Keywords: Patulin; Fermented feeds; Ruminal fermentation; Continuous culture

1. Introduction

Corn silage and high moisture corn are fermented feeds frequently used in beef and dairy rations in the USA. A multitude of factors, including molds, may adversely affect the quality of fermented feeds. Molds identified in fermented feeds include *Aspergillus* sp., *Cladiosporum* sp., *Fusarium* sp., *Mucor* sp., *Penicillium* sp., and adverse effects of molds may occur either through their deleterious effects on nutrient quality and/or their production of mycotoxins.

Penicillium sp. are commonly found in fermented feeds and are known to produce several mycotoxins including citrinin, ochratoxin, roquefortine, PR toxin, penicillic acid and patulin (Pelhate, 1977; Seglar, 1999). Patulin contamination of silage has been associated with hemorrhagic disorders in cattle in England (Syret, 1977). Other patulin-related diseases have been reported in Japan, France, and Germany when cattle ingested moldy fermented feeds (Hori et al., 1954; Moreau and Moreau, 1960; Jacquet et al., 1963; Schultz et al., 1968).

Patulin (4-hydroxy-4H furo (3,2C)pyran- 2(6HO)-one) is toxic to a wide range of organisms including microbes, plants, and animals (Singh, 1967; Stott and Bullerman, 1975; McKinley and Carlton, 1991). Patulin has an antimicrobial effect on aerobic Gram-positive and Gram-negative bacteria and also affects anaerobic bacteria (Singh, 1967). Acetic acid production was reduced in a batch culture exposed to 100 μg/ml of patulin (Escuola, 1992). In vitro dry matter (DM) and organic matter (OM) digestibilities were reduced in wheat straw to which 10 nmol of patulin had been added (Abdelhamid et al., 1992), we found that high doses of patulin adversely affect fermentation by ruminal microbes maintained in continuous culture fermenters (Tapia et al., 2002).

In this study, we tested the hypotheses that patulin-producing *Penicillium* sp. molds occur naturally in Upper Midwest (USA) corn silage and high moisture corn and that low doses of patulin adversely affect ruminal fermentation.

2. Materials and methods

2.1. Experiment 1. Do patulin-producing Penicillium sp. molds occur naturally in Upper Midwest (USA) fermented feeds?

2.1.1. Feed samples and mycological analysis

Fifty-one samples of fermented corn silage and high moisture-corn submitted for mold counts to the Veterinary Diagnostic Laboratory at the University of Minnesota (St. Paul, MN, USA) and 32 samples of fermented feeds submitted to the Dairy Herd Improvement Association (DHIA) Central Laboratories in Sauk Centre (MN, USA) were cultured for mold identification.

Fifty grams of fresh feed were homogenized with 450 ml (1 mg/g) sterile peptone buffer for 2 min in a blender, resulting in a 1:10 dilution. Further dilutions were prepared by transferring 1 ml aliquots of the initial dilution into succeeding dilution bottles containing 9 ml of peptone buffer. One ml portions of these dilutions were placed on acid potato dextrose agar (APDA) in Petri plates, which were incubated in the dark at 22–25 °C for 5 days. Mold colonies were identified to the genus level on the basis of their culture and morphology (Raper and Thom, 1949; Piit, 1979). The number of colony forming units per gram (cfu/g) of feed was determined for each genus of mold. The patulin-producing potential of the *Penicillium* sp. isolates was determined.

2.1.2. Patulin production and analysis

Conidium suspensions of the *Penicillium* sp. isolates were then grown on Czapeck yeast autolysate (CYA) slants and incubated for 7 days at 22–25 °C. Conidium suspensions made from these slants were cultivated in the dark on potato dextrose (PD), yeast extract sucrose (YES) and CYA agar plates at 22–25 °C for 14 days. These media were selected on the basis of their established ability to produce Penicillium mycotoxins (Filterborg et al., 1996).

The agar culture was extracted using chloroform:methanol (2:1). The organic phase was filtered through Phase Separation filter paper and evaporated in a water bath under a N_2 stream. The residue was redissolved in chloroform:methanol. Aliquots of the organic extract were analyzed for patulin by TLC, then HPLC. The TLC plates were developed in toluene:ethyl acetate:formic acid (5:4:1). Patulin was visualized by spraying the plate with methylbenzothiazolone hydrochloride and heating for 15 min at 100 °C. Patulin appeared as a yellow fluorescent spot under long wavelength UV (366 μm) light. HPLC analysis of patulin was carried out using a Rabbit HP (Rainin Instrument Co., Woburn, MA, USA) HPLC pump; a Spectroflow 757 UV absorbance detector; and a 3392A Integrator (Hewlett Packard, Palo Alto, CA, USA). The column was a 150 mm × 4.60 mm reversed phase LUNA 5 μm C_8 (Phenomenex, Torrance, CA, USA). The mobile phase was water:acetonitrile (95:5) with a flow rate of 1 ml/min. For injection into the HPLC, the organic extract was evaporated to dryness under a N_2 stream, reconstituted in methanol, and filtered through 0.45 μm filters. Patulin was detected by UV absorbance at 276 nm. Colonies were reported as either having, or not having, patulin-producing potential.

2.2. Study 2. Effects of patulin on fermentation by ruminal microbes in continuous culture fermenters

2.2.1. Patulin source

Patulin was produced by growing *Penicillium griseofulvum* NRRL 5256 on Difco® potato dextrose broth (Becton Dickinson Co., NJ, USA) at 28 °C for 4 weeks. The mycelia and broth were extracted twice with ethyl acetate. Extracts were pooled, evaporated to dryness in a rotary evaporator, and reconstituted in chloroform. Extracts were kept at −18 °C until used. Immediately prior to use, aliquots of the chloroform extract were evaporated to dryness under a N_2 stream, reconstituted in methanol, filtered through a 0.45 μm filter, and quantitated by HPLC. Chloroform extracts were evaporated to dryness and then reconstituted in distilled water immediately before addition to the continuous culture fermenters.

2.2.2. Continuous culture system and operation

Each experiment was conducted in two 7-day periods, with 4 days for stabilization and 3 days for patulin addition and sample collection. Eight single flow continuous culture fermenters (Hannah et al., 1986) were inoculated with ruminal fluid from a canulated cow fed a 70:30 (DM basis) forage:concentrate diet. Fermenters were provided with 75 g of DM/day of a pelleted diet in eight equal "meals" by an automated feeding device. The pelleted diet contained 390 g alfalfa hay, 280 g corn silage, 270 g cracked corn, 50 g soybean meal, and 6 g mineral mix/kg diet (DM basis). The chemical composition of the pelleted diet was: 925 g OM, 148 g crude protein (CP), 283 g neutral detergent fiber (NDF), 160 g acid detergent fiber (ADF), 276 g total non-structural carbohydrates (TNC)/kg (DM basis). Flow rate of each fermenter was set at 0.06 h^{-1} by regulating the buffer input. The culture pH was recorded every 10 min by an electronic data acquisition system (Daisy Lab®, National Instrument Services, TX, USA) and was maintained between 5.8 and 6.2 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions in fermenters were maintained by continuous infusion of N_2. The fermenter temperature was maintained at 38.5 °C. Two fermenters were supplemented with 0, 10, 20 or 40 mg of patulin in 1 ml of water every 12 h for the last three consecutive days of each period.

2.2.3. Sample collection and analytical procedures

During the sampling period, fermenter effluents were maintained at 2 °C in a water bath to retard microbial and enzymatic activities. Fermenter effluents were homogenized, and three separate 500 ml aliquots were removed daily and composited by fermenter. Composite effluent samples were kept at −20 °C until analysis for total N, ammonia N (NH_3-N), and volatile fatty acids (VFA).

Freeze-dried composite samples were analyzed for DM, OM, NDF, ADF, TNC, ash and purines. At the end of each experimental period, the contents from each fermenter were strained through two layers of cheesecloth, centrifuged at $1000 \times g$ for 10 min to remove feed particles then the supernatant was centrifuged at $20{,}000 \times g$ for 20 min to separate bacteria. Bacterial pellets were resuspended in distilled water, frozen and lyophilized. Purine concentrations were determined by the method of Zinn and Owens (1986). Purine contents of effluents and bacteria were used to partition effluent N flow into microbial and dietary N. Total N in the effluent, bacteria, and diet was determined by a Kjeldahl method (AOAC,

1984; ID 954.01). NH_3-N was determined by steam distillation using a Kjeltech 2300 Analyzer Unit (Tecator, Herdon, VA, USA). Effluent VFA concentrations were measured by gas chromatography (Hewlett Packard, model 5880A, Palo Alto, CA, USA) with a Carbopack DA/0.3% Carbowax 20M column (Supelco, Bellefonte, PA, USA). Sequential detergent fiber analyses (Van Soest et al., 1991) were used to determine NDF and ADF concentrations of the diet and effluents with heat stable amylase and sodium sulfite used in the NDF procedure. TNC in freeze-dried composite samples were determined enzymatically using ferricyanide as a colorimetric indicator (Smith, 1969).

2.2.4. Statistical

Data from the in vitro fermentation study were analyzed as a randomized complete block design using the GLM procedure of SAS (1989). The linear model used for each dependent variable was:

$$Y_{ij} = \mu + P_i + T_j + \varepsilon_{ij}$$

where μ: common mean, P_i: period (block), T_j: treatment, and ε_{ij}: the random error. Polynomial linear and quadratic contrasts were used to partition treatment sums of squares.

3. Results

3.1. Study 1. Identification of patulin-producing Penicillium sp. as fermented feed contaminants

Eleven genera of filamentous molds were isolated from the feed samples submitted to the Veterinary Diagnostic Laboratory. Samples provided by DHIA were known to be positive for *Penicillium* sp., which were isolated from 0.82 of the samples. Other isolated molds (Table 1) were: *Mucor* sp. (0.45), *Aspergillus* sp. (0.41), *Fusarium* sp. (0.25), *Cladosporium*

Table 1
Isolated molds and mold colony forming units cfu/g of feed from samples of fermented feeds submitted to the Veterinary Diagnostic Laboratory (UMN)

Mold	Samples	(cfu)/g				
		10^2	10^3	10^4	10^5	$>10^6$
Penicillium sp.	42	7	9	11	13	2
Mucor sp.	23	8	5	5	4	1
Aspergillus sp.	21	2	9	7	1	2
Fusarium sp.	13	3	5	3	1	1
Cladosporium sp.	8	2	2	3	1	0
Alternaria sp.	6	1	1	3	1	0
Monascus sp.	4	0	1	1	1	1
Rhizopus sp.	2	1	1	0	0	0
Phoma sp.	2	0	0	2	0	0
Scopulariosis sp.	1	0	0	0	1	0
Trichoderma sp.	1	1	0	0	0	0

sp. (0.16), *Alternaria* sp. (0.11), *Monascus* sp. (0.08) *Rhizopus* sp. (0.04), *Phoma* sp. (0.04), *Scopulariosis* sp. (0.02), *Trichoderma* sp. (0.02). In addition to *Penicillium* sp., 10 other fungal genera were isolated from the feed samples provided by the DHIA Laboratory. *Aspergillus* sp. (0.25), *Mucor* sp. (0.18), *Rhizopus* sp. (0.18) and *Fusarium* sp. (0.18) were the fungi most commonly isolated.

Of the feed samples submitted to the Veterinary Diagnostic Laboratory (UMN), 0.61 had total fungal contaminations higher than 10^5 cfu/g, and 0.52 of these samples had more than 10^4 cfu/g of *Penicillium* sp.

Of the *Penicillium* sp. isolates, 0.03 produced detectable amounts of patulin when cultured on YES and PD agar under laboratory conditions. These *Penicillium* molds belonged to the *P. viridicatum* group.

3.2. Study 2. Effects of patulin on fermentation by ruminal microbes in continuous culture fermenters

Apparent and true OM digestion decreased linearly ($P < 0.05$) with increasing patulin concentration (Table 2). Although NDF and ADF digestion decreased linearly ($P = 0.001$) with increasing patulin concentration, a quadratic relationship was also observed ($P < 0.05$). TNC digestion decreased linearly ($P < 0.05$) as patulin concentration increased.

The pH increased linearly ($P < 0.05$) in fermenters dosed with patulin. As a result, lower amounts of NaOH were needed to maintain the pH between 5.8 and 6.2 (Fig. 1).

Total and non-ammonia N (NAN) flows were not affected by patulin (Table 3). CP digestion and bacterial N flows decreased linearly ($P < 0.05$) as patulin increased. Conversely, there was a linear increase ($P < 0.05$) in NH_3-N flow and concentration with increased patulin. The decrease in bacterial N flow correlated with a greater (linear, $P < 0.05$) concentration of dietary N outflow in patulin-treated fermenters.

Efficiency of microbial protein synthesis (EMPS), was not affected by patulin but there was a linear decreased ($P < 0.05$) in the efficiency of N utilization (ENU).

Total VFA concentration (mol/l) decreased linearly ($P < 0.001$) with increasing patulin (Table 4). Molar proportions of acetate and propionate (mmol/mol) showed a quadratic

Table 2
The effects of different concentrations (mg/l) of patulin on pH and digestibility in continuous culture fermenters

	Patulin concentrations (mg/l)					Contrasts (*P*)	
	0	10	20	40	MSE	Linear	Quadratic
pH	5.83	5.85	5.87	5.94	0.05	0.016	0.354
Apparent OM	0.349	0.328	0.300	0.295	0.033	0.026	0.640
True OM[a]	0.472	0.432	0.391	0.377	0.039	0.003	0.520
NDF	0.317	0.154	0.93	0.113	0.068	0.001	0.023
ADF	0.536	0.398	0.323	0.358	0.060	<0.001	0.014
TNC	1.000	0.894	0.923	0.873	0.067	0.040	0.433

[a] Corrected for contribution of bacterial OM in the effluent.

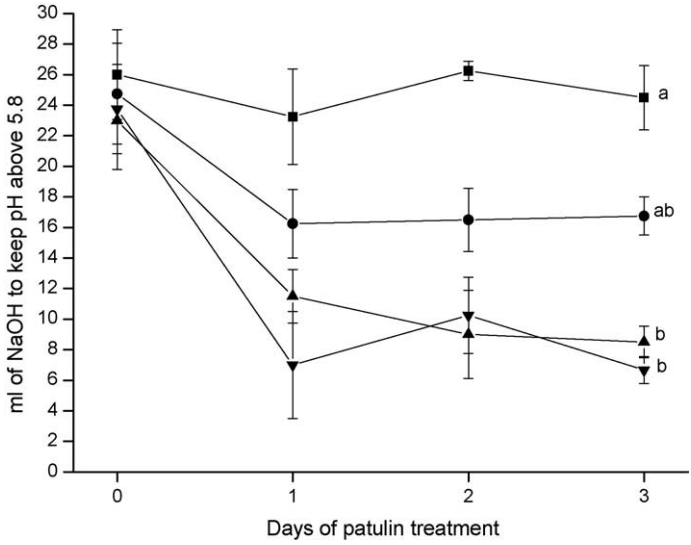

Fig. 1. Effect of 0 ppm (■), 10 ppm (●), 20 ppm (▲) and 40 ppm (▼) of patulin on the volume of NaOH required to keep the pH above 5.8 in continuous culture fermenters. Error bars represent the standard error of the mean.

effect ($P = 0.006$ and $P < 0.05$, respectively) (Table 4). A quadratic effect was also observed on the acetate:propionate ratio ($P < 0.05$). Lactate concentration (mmol/l) increased from 0.0 to 216.5 mmol/l (linear, $P < 0.05$) with increasing patulin concentration. The molar proportions of butyrate, valerate and branched-chain VFA (BCVFA) were unchanged.

Table 3
The effects of different concentrations (mg/l) of patulin on nitrogen metabolism by ruminal microbes in continuous culture fermenters

	Patulin concentrations (mg/l)					Contrasts (*P*)	
	0	10	20	40	MSE	Linear	Quadratic
N intake (g/day)	2.49	2.50	2.50	2.49	0.01	0.357	0.169
N flow (g/day)							
Total	1.94	1.98	1.95	1.93	0.08	0.693	0.492
Ammonia	0.04	0.04	0.07	0.09	0.03	0.026	0.362
Non-ammonia	1.90	1.95	1.88	1.86	0.09	0.356	0.458
Bacterial	0.70	0.60	0.50	0.43	0.12	0.005	0.817
Dietary	1.19	1.34	1.37	1.41	0.11	0.031	0.302
NH_3-N concentration (mg/l)	27.0	22.1	41.5	53.9	4.8	0.045	0.253
CP digestibility	0.457	0.390	0.376	0.362	0.048	0.031	0.302
Bacterial N (g/kg DM)	51.6	55.0	55.0	53.7	3.8	0.469	0.262
EMPS[a]	21.34	19.89	18.59	16.59	3.71	0.088	0.887
ENU[b]	0.54	0.52	0.46	0.41	0.08	0.035	0.772

[a] EMPS: efficiency of microbial protein synthesis (g of bacterial N/kg of OM truly fermented in continuous culture).

[b] ENU: efficiency of N utilization by ruminal bacteria [(g of microbial nitrogen/g of ruminal available nitrogen) × 100].

Table 4
The effect of different concentrations (mg/l) of patulin on volatile fatty acid concentrations in continuous culture fermenters

	Patulin concentrations (mg/l)					Contrasts	
	0	10	20	40	MSE	Linear	Quadratic
Total VFA (mmol/l)	171.0	145.6	124.7	109.2	13.5	<0.001	0.481
Individual VFA (mmol/mol)							
Acetate (mmol/mol)	516.2	415.4	432.1	498.3	48.4	0.733	0.006
Propionate (mmol/mol)	321.6	372.4	353.4	310.2	41.7	0.553	0.039
Butyrate (mmol/mol)	119.6	154.3	157.4	136.1	29.0	0.410	0.086
BCVFA (mmol/mol)	12.5	9.7	7.5	8.2	4.5	0.120	0.525
Valerate (mmol/mol)	30.0	47.3	49.4	47.1	12.1	0.075	0.136
Lactate (mmol/l)	0.0	3.1	44.2	216.5	36.6	0.029	0.200
Acetate:propionate	1.6	1.1	1.2	1.6	0.3	0.767	0.014

4. Discussion

4.1. Study 1. Identification of patulin-producing Penicillium sp. as fermented feed contaminants

Our results agree with a previous report indicating that *Mucor*, *Penicillium*, *Aspergillus* and *Monilia* were the most prevalent molds isolated from animal feeds in the USA (Seglar, 1999). Similar findings have been reported for fungal isolation from fermented feed in Europe. In one survey, 22 genera of filamentous molds were isolated from corn, sorghum and gramineous silage. Of the fermented feed samples, 0.82 contained *Penicillium* molds. *Mucor* sp. (0.52) and *Aspergillus* sp. (0.48) were the second and third most abundant molds, respectively. *Penicillium cyclopium* (0.20), *P. granulatum* (0.20), *P. claviforme* (0.10) and *P. frequentans* (0.10) were the *Penicillium* strains more commonly isolated (Escuola et al., 1972).

In Bulgaria, molds of the genera *Mucor*, *Penicillium*, *Aspergillus*, *Alternaria* and *Trichoderma* were commonly demonstrated in a survey of 1050 samples taken at various depths from maize silage (Krustev and Kristov, 1981).

During 2000, 53 corn silage samples and high moisture corn samples from Argentina were evaluated using the same techniques. *Penicillium* sp. (0.35) and *Aspergillus* sp. (0.27) were the filamentous molds most commonly isolated (Tapia, unpublished). Other isolated molds include: *Byssochlamys* sp., *Cladosporium* sp., *Fusarium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizopus* sp.

In the present study, 0.61 of the feed samples had more than 10^5 cfu/g and 0.52 of the *Penicillium* sp. isolates had more than 10^4 cfu/g. According to Seglar (1999), silages are heavily infested with molds when total mold populations exceed 10^5 cfu/g of feed silage. This level of fungal contamination may affect feed by diminishing its nutrient value, by decreasing palatability, or by the adverse effect of mycotoxins on animal health.

The media used for patulin production by *Penicillium* sp. were selected on the basis of their established ability for producing mycotoxins (Filterborg et al., 1996). Of the *Peni-*

cillium sp. isolated in our study, 0.03 produced patulin. Higher proportions of positive patulin-producing molds have been obtained with *Paecilomyces* sp. isolated from corn silage (Anderson et al., 1979). In samples from Argentina (Tapia, unpublished), one out of eight strains of *Penicillium* sp. and one out of one *Byssochalmys* (*Paecilomyces* sp.) produced detectable amounts of patulin on YES agar.

Although results from in vitro cultures cannot necessarily be extrapolated to natural conditions, they do indicate the ability of patulin-producing fungi to grow and express toxicity under some conditions. The production and persistence of patulin in feeds is determined by the potential of the substrate to support toxin production and the stability of patulin in the substrate in which it has been produced. Stability studies of patulin in different substrates showed that substrates high in carbohydrate encourage mold growth and patulin production. Therefore, high moisture corn and corn silage might be good substrates for *Penicillium* growth and patulin production due to their high carbohydrate content.

Muller and Amend (1997), reported that patulin reached a high concentration, and then decreased to a low level, when *P. roqueforti* was grown on maize silage. It is probable that under natural conditions the examination of a feedstuff for patulin may be negative due to its degradation, but it could still be possible to isolate the causative mold. This situation could be hazardous when the silage has been opened thereby allowing toxigenic molds to proliferate rapidly and produce patulin. Moreover, under natural conditions, the amount of patulin formed will depend on microbial interactions and other factors. It has been demonstrated that high counts of yeast controlled the amount of patulin formed by degrading it, but coincidentally high levels of patulin may also inhibit yeast proliferation (Dutton et al., 1984).

4.2. Study 2. Effects of patulin on fermentation by ruminal microbes in continuous culture fermenters

The purpose of this study was to determine adverse effects of increasing concentrations of patulin on microbial fermentation. There was a significant effect on the digestion of OM, NDF, ADF and TNC. These results are in agreement with the observations from Abdelhamid et al. (1992), who reported that 10 nmol of patulin reduced in vitro DM and OM digestion of wheat straw.

In vivo, ruminal pH homeostasis is primarily maintained by the buffering effect of saliva and the accumulation of organic acids in the rumen content. In this study, the pH was controlled by addition of acid or base solutions as needed, and by the artificial saliva flowing into the fermenters. Thus, a decrease in bacterial activity and a decrease in concentration of VFA resulted in an increase in pH and lower amount of base needed to keep the pH above 5.8.

Bacterial N flow was lower in fermenters supplemented with patulin. This result correlates with a greater dietary N flow observed in patulin-treated fermenters. Inhibition of bacterial protein synthesis when patulin was added to rumen microflora under batch culture conditions has been reported (Escuola, 1992).

There was an increase in NH_3-N flow and a decrease in CP degradation in effluents of fermenters treated with patulin. The main cellulolytic bacterial species use NH_3 as their main source of N for microbial protein synthesis, thus the NH_3-N concentration in fermenter

fluid depends on the extent of protein degradation and rate of N uptake by the microbes. The decrease in this bacterial population or in its efficiency of N utilization could lead to an accumulation on NH_3-N in the effluents. Similar results were obtained in a previous in vitro study with greater doses of patulin (Tapia et al., 2002). Other in vitro rumen fermentation studies using long-term rumen simulation (RUSITEC), demonstrated a 68 and 35% increase in NH_3-N concentrations for moldy corn silage and moldy grass, respectively (Maiworm et al., 1995; Holtershinken et al., 1997).

The concentrations of patulin used in the present study did not affect EMPS. However, there was a significant decrease in the bacterial efficiency to utilize available ruminal N. This result suggests that patulin not only affects microbial protein concentration, as seen by a low bacterial N flow but also may affect bacterial activity.

The total VFA production was severely affected by patulin. These changes were consistent with a low digestibility of OM and may be a consequence of a decrease in bacterial populations. Holtershinken et al. (1997) reported a reduction of 9.9 and 12.2% in the production of acetate and propionate, respectively, and an increase of up to 39.9% in *n*-butyrate in continuous culture of ruminal fluid obtained from a cow fed moldy grass. In the present study, molar proportions of acetate and propionate showed a quadratic effect. Intermediate concentrations of patulin (10 and 20 mg/l) produced a decrease in acetate proportion and an increase propionate proportion. Accordingly, the acetate:propionate ratio decreased with 10 and 20 mg/l of patulin but it was unchanged in fermenters dosed with 40 mg/l of patulin. The decrease in acetate molar proportion may be attributed to a modification in the cellulolytic bacterial population, consistent with low digestibilities of NDF and ADF. At higher concentration of patulin (40 mg/l), there was also an effect on propionate forming bacteria. The reduction in the activity of these bacteria may be the reason for the increase in lactate concentration in patulin fermenters. Lactate is a preferred substrate of propionate-forming bacteria (Gottschalk, 1986), therefore it is expected that an inhibition in the activity of these bacteria may result in a decrease of propionate and consequently an accumulation of lactate.

5. Conclusions

This study demonstrates that *Penicillium* sp. molds are common contaminants of fermented corn silage and high moisture corn in the Upper Midwest (USA), that some of these *Penicillium* strains have the capacity to produce patulin when cultured under laboratory conditions, and that patulin has the potential to adversely affect rumen fermentation.

The in vitro rumen fermentation studies demonstrated that concentrations of 10–40 ppm of patulin affected both digestion of OM and fiber and the production of bacterial end products, such as VFA and microbial protein. It is well known that alterations in the rumen digestion of DM and production of microbial end products can impact the production and/health of the ruminant animal.

Further surveys for the detection of patulin and other *Penicillium* mycotoxins in silage are needed to determine the prevalence of these mycotoxins in other common animal feeds. An understanding of the conditions leading to *Penicillium* growth and mycotoxin production in fermented feeds is also needed to develop proper practices for silage management.

References

Abdelhamid, A.M., El-Ayouty, S.A., El-Saadany, H.H., 1992. The influence of contamination with separate mycotoxins (aflatoxins, ochratoxin A citrinin, patulin, penicillic acid or sterigmatocystin) on the in vitro dry matter and organic matter digestibilities of some roughages (Berseem hay and wheat straw). Arch. Anim. Nutr. 42, 179–185.

Anderson, M., Dutton, M., Harding, K., 1979. Production and degradation of patulin by *Paecilomyces* species, a common contaminant of silage. J. Sci. Food Agric. 30, 229–232.

Association of Official Analytical Chemists, 1984. Official Methods of Analysis, 14th ed. AOAC, Arlington, VA, USA.

Dutton, M.F., Weslake, K., Anderson, M.S., 1984. The interaction between additives, yeasts and patulin production in grass silage. Mycopathologia 87, 29–33.

Escuola, L., 1992. Patulin production by *Penicillium granulatum* and inhibition of ruminal flora. J. Environ. Pathol. Toxicol. Oncol. 11, 109–112.

Escuola, L., Le Bars, J., Larrieu, G., 1972. Etudes sur la mycoflore des ensilages mycoflore des fronts de coupe d'ensilages de graminees fourrageres. Ann. Rech. Vet. 3, 469–481.

Filterborg, O., Frisvad, J.C., Thrane, U., Lund, F., 1996. Screening methods for secondary metabolites produced by fungi in pure cultures. In: Samsom, R.A., Hoesktras, E.S., Frisvad, J.C., Filterborg, O. (Eds.), Introduction to Food-borne Fungi. Centralalbureau Voor Schimmelcultures, Baarn, The Netherlands, pp. 270–274.

Gottschalk, G., 1986. Bacterial fermentations. In: Gottschalk, G. (Ed.), Bacterial Metabolism, second ed. Springer-Verlag, NY,USA, pp. 208–282, Chapter 8.

Hannah, S.M., Stern, M.D., Ehle, F.R., 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of fixed diets containing various soy bean products. Anim. Feed Sci. Technol. 16, 51–62.

Holtershinken, M., Plitt, U., Tammen, F., Hoffmann, C.P., Scholz, H., 1997. Influence of moldy grass on fermentation and thiamine metabolism in bovine rumen fluid (in-vitro). Dtsch. Tierarztl. Wochenschr. 104, 17–22.

Hori, M., Yamamoto, T., Ozawa, A., Matzuki, Y., Hamaguchi, A., Soraoka, H., 1954. Studies on a fungus species isolated from the malt-feed which caused mass death of cows. Jpn. J. Bacteriol. 9, 1105–1111.

Jacquet, J., Boutibonnes, P., Cicile, J.P., 1963. Observations sur la toxicite d' *Aspegillus clavatus* pour les animaux. Bull. Acad. Vet. 32, 119–207.

Krustev, E., Kristov, B., 1981. The mycoflora in maize silage. Vet. Nauki. 18, 88–91.

Maiworm, V.K., Holtershinken, M., Scholz, H., 1995. The effects of mouldy and decomposed silage on rumen. Tierarztl. Umsch. 50, 283–290.

McKinley, E.R., Carlton, W.W., 1991. Patulin. In: Sharma, R.P., Salunkhe, D.K. (Eds.), Mycotoxins and Phytoalexins. CRC, Boca Raton, FL, pp. 191–236.

Moreau, C., Moreau, M., 1960. Un danger pour le betail nourri de plantules fourrageres cultivees en germoirs: lapullulation d'une moisissure toxique, l'*Aspergillus clavatus*, cause des accidents mortels. C.R. Seances Acad. Agric. Fr. 46, 441–445.

Muller, H.M., Amend, R., 1997. Formation and disappearance of mycophenolic acid, patulin, penicillic acid and PR toxin in maize silage inoculated with *Penicillium roqueforti*. Arch. Anim. Nutr. 50, 213–225.

Pelhate, J., 1977. Maize silage: incidence of moulds during conservation. Folia Vet. Lat. 7, 1–16.

Piit, J.I., 1979. The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London, UK.

Raper, K.B., Thom, C., 1949. A Manual of the Penicillia. The Williams and Wilkins Co., Baltimore.

SAS Institute Inc., 1989. SAS/STAT User's Guide, Version 6, Vol. 2, fourth ed. SAS Inst. Inc., Cary, NC, USA.

Schultz, V.J., Motz, R., Schafer, M., Aumgart, W., 1968. Experimentelle untersuchungen zur *Aspergillus-clavatus*-vergiftung beim rind. Monatschr. Vet. 24, 14–17.

Seglar, B., 1999. Molds and Mycotoxins in Ensiled Forages. (INTERNET) Pioneer Hi-Bred Intl. Inc., Johnston, IA, USA, p. 16.

Singh, J., 1967. Patulin. In: Gottlieb, D., Shaw, P.D. (Eds.), Antibiotics: Mechanism of Action, Vol. 1. Springer-Verlag, Berlin, Germany, pp. 621–630.

Smith, D., 1969. Removing and analysing total non-structural carbohydrates from plant tissue. Wisconsin Agric. Exp. Stat. Rep. 41, 1–11.

Stott, W.T., Bullerman, L.B., 1975. Patulin: a mycotoxin of potential concern in foods. J. Milk Food Technol. 38, 695–705.

Syret, R., 1977. Moulds and mycotoxins in animal foodstuffs—reports of ADAS microbiologists, pp. 4–7.

Tapia, M.O., Stern, M.D., Koski, R.L., Bach, A., Murphy, M.J., 2002. Effects of patulin on rumen microbial fermentation in continuous culture fermenters. Anim. Feed Sci. Technol. 97, 239–246.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Zinn, R.A., Owens, P.N., 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. 66, 157–166.